

Identification of Peroxiredoxin-1 as a Novel Biomarker of Abdominal Aortic Aneurysm

Roxana Martinez-Pinna, Priscila Ramos-Mozo, Julio Madrigal-Matute, Luis M. Blanco-Colio, Juan A. Lopez, Enrique Calvo, Emilio Camafeita, Jes S. Lindholt, Olivier Meilhac, Sandrine Delbosc, Jean-Baptiste Michel, Melina Vega de Ceniga, Jesus Egido, Jose L. Martin-Ventura

Objective—In the search of novel biomarkers of abdominal aortic aneurysm (AAA) progression, proteins released by intraluminal thrombus (ILT) were analyzed by a differential proteomic approach.

Methods and Results—Different layers (luminal/abluminal) of the ILT of AAA were incubated, and the proteins released were analyzed by 2-dimensional difference in-gel electrophoresis. Several differentially expressed proteins involved in main AAA pathological mechanisms (proteolysis, oxidative stress, and thrombosis) were identified by mass spectrometry. Among the proteins identified, peroxiredoxin-1 (PRX-1) was more released by the luminal layer compared with the abluminal layer of the ILT, which was further validated by Western blot, ELISA, and immunohistochemistry. We demonstrated increased PRX-1 serum levels in AAA patients compared with healthy subjects and also positive correlation among PRX-1 and AAA diameter, plasmin-antiplasmin, and myeloperoxidase levels. Finally, a prospective study revealed a positive correlation between PRX-1 serum levels and AAA expansion rate. Moreover, the combination of PRX-1 and AAA size had significantly additive value in predicting growth.

Conclusion—Several proteins associated with AAA pathogenesis have been identified by a proteomic approach in ILT-conditioned medium. Among them, PRX-1 serum levels are increased in AAA patients and correlate with AAA size and growth rate, suggesting the potential use of PRX-1 as a biomarker for AAA evolution. (*Arterioscler Thromb Vasc Biol.* 2011;31:935-943.)

Key Words: aneurysms ■ antioxidants ■ proteomics

Abdominal aortic aneurysm (AAA) is an important health problem, which occurs in up to 9% of adults older than 65 years of age. The incidence of asymptomatic and ruptured AAA has increased during recent decades, causing ≈1% to 2% of male deaths in Western countries.¹ Because AAAs are usually asymptomatic before rupture, the present clinical challenges are to diagnose AAA at an early stage and to decipher the biological mechanisms leading to progressive dilatation and finally rupture, to develop new diagnostic and therapeutic approaches. Identification of biomarkers could help to target both objectives.

Previous studies have identified AAA biomarkers by studying the levels of different molecules potentially related to AAA pathological mechanisms.^{2,3} A different noncandidate biomarker strategy using a set of modern high-throughput technologies, including proteomics, will offer new opportunities to gain a deeper insight into disease processes, including their molecular mechanisms,

the risk factors involved, and the analysis of disease progression.^{4,5} We have previously reported a differential proteomic approach to identify new atherothrombosis biomarkers released by the arterial wall into plasma using normal and pathological arteries in culture.^{6,7} The presence of an intraluminal thrombus (ILT) is a main feature of AAA, and recent data suggest that the biological activities of ILT play a major role in AAA development in humans.⁸ In this study, different layers (luminal/abluminal) from the ILT of human AAA patients were incubated in protein-free medium, and the released proteins were analyzed by a gel-based (2-dimensional difference in-gel electrophoresis [2D-DIGE]) proteomic approach followed by protein identification by mass spectrometry (MS). We focused on peroxiredoxin-1 (PRX-1) in view of its potential role in modulating oxidative stress and thus validated its differential expression by Western blot and ELISA in the conditioned medium. Finally, the suitability of PRX-1 as a

Received on: August 9, 2010; final version accepted on: January 12, 2011.

From the Vascular Research Laboratory, Instituto de Investigación Sanitaria-Fundación Jiménez Díaz-Autónoma University, Madrid, Spain (R.M.-P., P.R.-M., J.M.-M., L.M.B.-C., J.E., J.L.M.-V.); Unidad de Proteómica, Centro Nacional de Investigaciones Cardiovasculares, Madrid, Spain (J.A.L., E. Calvo, E. Camafeita); Vascular Research Unit, Viborg Hospital, Viborg, Denmark (J.S.L.); Institut National de la Santé et de la Recherche Médicale U698, Paris, France (O.M., S.D., J.-B.M.); Université Denis Diderot, Paris, France (O.M., S.D., J.-B.M.); Assistance Publique Hôpitaux de Paris, Centre Hospitalier Universitaire, Bichat-Claude Bernard, Paris, France (O.M., S.D., J.-B.M.); Vascular Surgery, Hospital Galdakao, Bilbao, Spain (M.V.d.C.).

Correspondence to Jose Luis Martin-Ventura, PhD, Vascular Research Laboratory, Fundación Jiménez Díaz, Autónoma University, Av Reyes Católicos 2, 28040, Madrid, Spain. E-mail jlmartin@fjd.es

© 2011 American Heart Association, Inc.

Arterioscler Thromb Vasc Biol is available at <http://atvb.ahajournals.org>

DOI: 10.1161/ATVBAHA.110.214429

biomarker of AAA presence and progression was assessed in sera from 2 different population studies by ELISA.

Materials and Methods

AAA Tissue and Tissue-Conditioned Media

Ten human AAA thrombus samples were collected during surgical repair and dissected into luminal and abluminal parts (at the interfaces with circulating blood and with the remaining media, respectively). AAA samples were obtained from patients undergoing surgery, enrolled in the RESAA protocol (Reffet sanguin de l'évolutivité des anévrismes de l'aorte abdominale; Comités Consultatifs de Protection des Personnes dans la Recherche Biomédicale Paris-Cochin numbers 2095, 1930, and 1931).⁹ All patients gave their informed written consent, and the protocol was approved by a French ethics committee (Comités Consultatifs de Protection des Personnes dans la Recherche Biomédicale, Cochin Hospital). Luminal and abluminal layers of AAA thrombus were cut into small pieces (5 mm³) and separately incubated in RPMI 1640 medium containing antibiotics and an antimycotic (Gibco) for 24 hours at 37°C (6 mL/g of wet tissue). The conditioned medium (supernatant containing proteins released by the tissue sample) was obtained after centrifugation at 3,000g for 10 minutes at 20°C. The protein concentration of each conditioned medium was measured using the Bradford assay (Bio-Rad).

Sample Preparation and DIGE Protein Labeling

Supernatants from luminal and abluminal layers of ILT human samples from 4 individual patients were isolated, and the proteins were precipitated using the 2D clean-up kit (GE Healthcare) and resuspended in 30 mmol/L Tris-HCl, pH 8.5, 7 mol/L urea, 2 mol/L thiourea, and 40 g/L CHAPS. Protein concentration was measured by RC-DC protein assay kit (Bio-Rad).

The comparison between the proteins released by luminal and abluminal layers was performed by 2D-DIGE analyses across 4 gels, using the same pooled-sample internal standard, the equimolecular mixture of all the samples, in all gels. The samples were labeled according to manufacturer's instructions for minimal labeling, using 400 pmol of dye reagent for 50 µg of protein extract. Individual samples were labeled with Cy3 or Cy5 dyes using dye switching, and the internal standard was always Cy2 labeled. The labeling reaction was performed on ice for 30 minutes in darkness and quenched by 1 µL of lysine (10 mmol/L) for 10 minutes.

2D Electrophoresis and Image Acquisition

The 4-paired samples of Cy3- and Cy5-labeled proteins were mixed with 50 µg of Cy2-labeled internal standard. The mixtures were diluted in rehydration buffer (7 mol/L urea, 2 mol/L thiourea, 40 g/L CHAPS, 0.8% IPG buffer 3-11NL, and bromphenol blue) containing 50 mmol/L immobilized pH gradient and resolved on 24-cm pH 3-11 nonlinear gradient IPG strips (GE Healthcare). The samples were applied by cup loading to the previously rehydrated IPG Strips with 450 µL of the aforementioned rehydration buffer containing 97 mmol/L DeStreak (GE Healthcare).

The isoelectric focusing was carried out in an IPGphor II isoelectric focusing system (GE Healthcare) until a total of 42 kVh was reached. After isoelectric focusing, strips were equilibrated in buffer containing 6 mol/L urea, 400 mmol/L glycerol, 7 mmol/L SDS, and bromphenol blue, for 15 minutes with addition of 6.5 mmol/L dithiothreitol, and then without dithiothreitol but with the same buffer supplemented with 21.6 mmol/L iodoacetamide for an additional 15 minutes. SDS-PAGE was carried out on 12% polyacrylamide gels at 2 W/gel.

The differentially labeled coresolved proteins in each gel were acquired with a Typhoon 9400 laser scanner (GE Healthcare), and Cy2-, Cy3-, and Cy5-labeled images of each gel were acquired at excitation/emission wavelength values of 488/520, 523/580, and 633/670 nm, respectively. Finally, gels were fixed in 12% methanol and 7% acetic acid and silver stained using the Plus One silver staining kit (GE Healthcare).

DIGE Data Analysis

The images were analyzed using the DeCyder version 6.5 software (GE Healthcare) for spot detection and quantification and intergel matching and statistics. DeCyder calculates the average abundance for each spot among the 4 gels under study. Statistical significance was assessed for each change in abundance using the paired Student *t* test analysis. We considered spots present in all of the 12 images (Cy2-, Cy3-, and Cy5-labeled images of each gel) with statistical significance at 95% confidence level for standardized average spot volume ratios greater than 1.5.

Protein Identification by Matrix-Assisted Laser Desorption Ionization-MS

Differentially expressed spots were selected from silver-stained gels for gel excision, automated digestion,¹⁰ and analysis in an Ultraflex matrix-assisted laser desorption ionization (MALDI) time-of-flight/time-of-flight mass spectrometer (Bruker Daltonik) to obtain the corresponding MALDI-MS and MALDI-MS/MS spectra as described.^{11,12} These MS and MS/MS combined data were used to search a nonredundant protein database (NCBIInr; $\approx 10^7$ entries; National Center for Biotechnology Information, Bethesda, MD) using the Mascot software (Matrix Science).¹³ Detailed information is included the Supplemental Methods, available online at <http://atvb.ahajournals.org>.

Western Blot

Equal amounts (50 µg of protein) of conditioned medium were loaded onto 12.5% polyacrylamide gels, electrophoresed, and transferred to nitrocellulose membranes. Then they were blocked with 7% milk powder in TBS-T for 1 hour and incubated overnight at 4°C at 1:500 with anti-peroxiredoxin antibody (Santa Cruz Biotechnology, goat polyclonal sc-23969). Then, the membranes were washed with TBS-T and incubated with anti-goat antibody (1:2000) for 1 hour at room temperature. After 4 washes, the signal was detected using the ECL chemiluminescence kit (GE Healthcare).

ELISA

The soluble concentration of PRX-1 in both conditioned media and serum was quantified with a commercial kit following the manufacturer's instructions (AbFrontier). The interassay and intraassay variability values were 9% and 6%, respectively.

Immunohistochemistry

AAA thrombus samples were fixed in 3.7% paraformaldehyde and embedded in paraffin. Immunohistochemistry was performed on 5-µm sections, using rabbit anti-peroxiredoxin 1 (ab-15571) at 1:100 overnight at 4°C as the primary antibody. The peroxidase LSAB+ system horseradish peroxidase kit (Dako), followed by Histogreen peroxidase substrate (AbCys SA), was used for detection. Sections were then counterstained with Nuclear Fast Red for 5 minutes at room temperature before being mounted using Eukitt medium. Control irrelevant rabbit immunoglobulins (Dako) were applied at the same concentrations as primary antibodies to assess nonspecific staining.

Red Blood Cell In Vitro Experiments

Blood was collected in EDTA-tubes from 6 healthy volunteers. The blood sample was centrifuged at 2500 rpm for 15 minutes to eliminate plasma, and then blood cells were diluted 1:1 in PBS (154 mmol/L NaCl, 10 mmol/L phosphate buffer, pH 7.4) and were separated by centrifugation in Ficoll-Paque (GE Healthcare). Finally, red blood cells (RBCs) and leukocytes were separated by 6% dextran sedimentation of the pellet. Erythrocytes were incubated with lipopolysaccharide (0.1 and 1 ng/mL) and H₂O₂ (5 and 500 µmol/L) for 30 minutes. The lysis of RBCs was achieved by hypotonic shock, using repetitive washes with distilled water, and finally by adding NaCl (3.5%). Membrane fractions were collected after centrifugation at 12 000 rpm for 10 minutes.

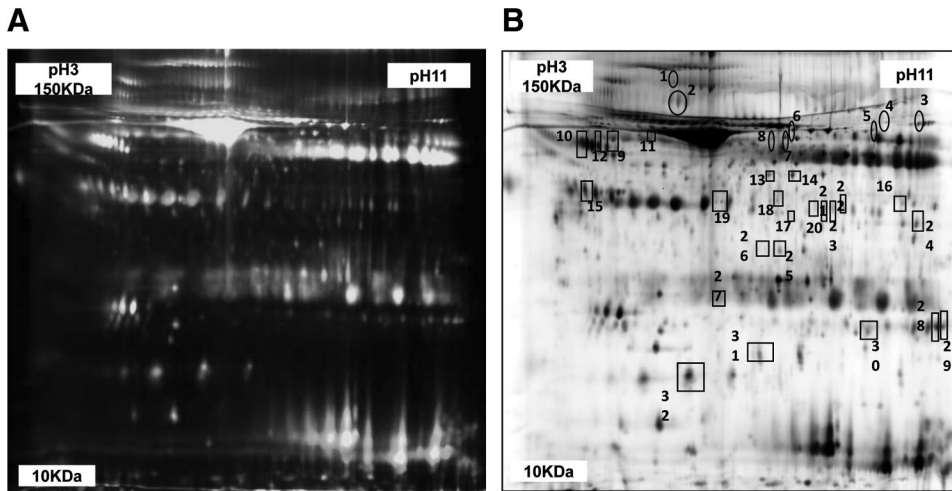


Figure 1. DIGE of AAA-conditioned media. Luminal and abluminal layers from ILT of 4 AAA patients were incubated in protein-free medium. Secreted proteins were labeled with the corresponding CyDye reagents, mixed, resolved on 4 independent DIGE gels, and imaged (red, Cy3; green, Cy5; blue, Cy2) and analyzed with DeCyder software. A, A representative gel image from the 3-plex DIGE experiment is shown. Proteins were resolved in the 3 to 11 (nonlinear) pH range in the first dimension and on 12% polyacrylamide gels in the second dimension. B, Spots showing statistically

significant regulation between the 2 conditions were excised from silver-stained gels and identified by MALDI-MS. Identified spots are numbered as listed in the Table.

Hydrogen Peroxide Quantification

Quantitative determination of hydrogen peroxide was performed with a commercial colorimetric kit (907-015, Assay Design) following the manufacturer's instructions.

AAA Patient Sera

Spanish Study

The study was approved by the Spanish center's research and ethics committees, and informed consent from the patients and the controls for their inclusion in the study was obtained. Eighty-three consecutive patients with an asymptomatic infrarenal AAA were recruited, and for each, the AAA size at the time of blood sample collection was registered (Supplemental Table I). We excluded patients with symptomatic or inflammatory AAA, multiple synchronic aneurysms (thoracic, femoral, popliteal), and AAA with a location other than infrarenal. We also excluded patients with active inflammatory or acute infectious processes, surgical procedures or major trauma in the previous 60 days, and chronic antiinflammatory or immunosuppressive medication.

Thirty-three controls were recruited from a screening program, which is currently being performed among the population in the area under our care.¹⁴ They were randomly selected from the screened individuals with nondilated (<30 mm, confirmed with abdominal ultrasound) infrarenal aortas. Neither patients nor controls showed any significant differences in age, sex, or cardiovascular risk factors.

Viborg Study

In 1994, half (4404) of all 65 to 73 year-old males in Viborg County, Denmark, were invited to B-mode-ultrasonographic screening for AAA at their regional hospital. The trial was approved by the respective local scientific ethics committees and reported to the Danish Central Control of Registers.

An AAA was defined as an infrarenal aortic diameter of 30 mm or more, and AAAs >50 mm were referred for surgery. AAAs of 30 to 49 mm were offered yearly follow-up examinations to check for any expansion.¹⁵ Two observers were used, and the arithmetic interobserver variation (2 SD) was 1.4 mm.¹⁶

The serum samples were left for coagulation at room temperature for 45 minutes before centrifugation. A random sample of 80 cases was used in this study of peroxiredoxin. Follow-up was truncated after 10 years. Sixteen men were referred to planned AAA repair because of expansion (Supplemental Table II). The expansion rate was calculated as the change in the anterior-posterior diameter during the whole observation period, transformed to annual units. The trial was approved by the respective local scientific ethics committees and reported to the Danish Central Control of Registers.

Statistics

ELISA and Western blot results are expressed as mean \pm SEM. The Wilcoxon paired test was used to analyze differences in PRX-1 levels between luminal and abluminal supernatants of the same samples. The analysis of small and large AAA and control groups was performed with nonparametric tests (Mann-Whitney *U* tests). Pearson correlation analysis was used to examine the univariate correlation between PRX-1 and AAA expansion rate, and it was adjusted for current smoking, body mass index, previous cardiovascular events, use of glucocorticoids, low-dose aspirin, β -blockers, angiotensin converting enzyme inhibitors, and ankle brachial blood pressure index. The receiving-operating characteristic (ROC) curve analysis was performed to test the predictive clinical value of PRX-1 and initial AAA size, univariately and combined in a multivariate linear regression model concerning prediction of cases expanding more than 1.68 mm/year (mean annual growth). For the analysis of the ROC curve, the null hypothesis was that the test had a performance similar to the diagonal line, ie, the area under the curve was 0.5. If the lowest 95% confidence limit for the area under the curve was more than 0.5, a significant predictive test was said to be present.

Results

DIGE Analysis of ILT Supernatants

Because ILT is involved in AAA evolution and ILT material can be sampled during surgery, it represents a highly relevant model for the study of biological events participating in human AAA. We hypothesized that the proteins differentially released by the biologically active luminal part of the ILT versus the abluminal layer may reflect ILT activity. Proteins released from these different layers (luminal/abluminal) of the ILT of AAA were analyzed by a noncandidate based proteomic strategy using 2D-DIGE (Figure 1). DeCyder analysis revealed 42 differentially expressed spots, and after silver staining of the gels, only visible spots were excised, digested with trypsin, and subjected to MS-based protein identification. Thirty-two proteins out of the 42 spots released differently by the luminal layer compared with the abluminal layer were finally identified by MS (Table and Supplemental Figure I); these proteins are involved in different AAA pathological mechanisms, such as redox balance (eg, peroxiredoxin), inflammatory processes (eg, complement compo-

Table. Proteins Differentially Released From Different Layer Regions (Luminal/Abluminal) of the ILT of Human AAA as Revealed by 2D-DIGE and Identified by MALDI-MS

DeCyder*			Protein Description†	Accession Code†	Mascot‡			Theoretical Molecular Mass (kDa)/pI	Matched Peptides§	Unmatched Peptides§	Coverage (%)§
Spot	Average Ratio	t Test			Total Score	Expected	Ion Score				
1	-2.57	3.81×10^{-2}	Complement component C3	gi 78101268	194	3.10×10^{-13}	NA	114.2/5.55	16	4	18
2	7.35	4.53×10^{-2}	Complement component C3	gi 78101268	230	7.80×10^{-17}	NA	114.2/5.55	20	3	23
3	-2.03	6.97×10^{-3}	Complement component C4A	gi 179674	133	3.90×10^{-7}	NA	189.1/6.7	11	1	7
4	-2.05	4.31×10^{-2}	Complement component C4A	gi 179674	444	5.30×10^{-40}	263	194.3/6.65	17	4	11
5	1.74	4.68×10^{-2}	Transketolase	gi 388891	300	7.70×10^{-24}	83	685.3/7.89	13	1	28
6	-1.96	2.82×10^{-4}	Complement component C3	gi 78101267	642	4.80×10^{-58}	341	71.3/6.82	18	2	43
7	-1.85	2.5×10^{-2}	Transketolase	gi 58176651	209	9.70×10^{-15}	98	56.8/7.16	8	3	22
8	-1.84	2.48×10^{-2}	Phosphoglucosyltransferase 1	gi 21361621	279	9.70×10^{-22}	71	61.7/6.3	13	3	29
9	-1.67	1.85×10^{-2}	Antithrombin III	gi 999513	474	3.10×10^{-41}	321	49.4/5.95	13	3	39
			IGHA1 protein	gi 16741036	272	4.80×10^{-21}	109	54.3/8.07	10	3	26
10	-1.65	8.42×10^{-3}	Prolyl 4-hydroxylase	gi 20070125	113	6.60×10^{-7}	77	57.5/4.76	2	0	6
11	-1.58	4.62×10^{-2}	Dihydropyrimidinase-like 2	gi 4503377	208	1.20×10^{-14}	69	62.7/5.95	9	3	20
12	-1.52	3.67×10^{-2}	Tubulin- β -2c	gi 14124960	148	2.10×10^{-10}	53	26/4.95	5	1	17
13	-1.97	2.38×10^{-2}	Hemopexin	gi 386789	152	8.30×10^{-11}	66	52.2/6.57	5	1	16
14	-1.81	1.27×10^{-2}	Enolase 1	gi 203282367	403	3.90×10^{-34}	213	47.4/6.99	13	4	41
15	-2.14	4.93×10^{-3}	Trypsin-like serine protease	gi 47124562	148	2.10×10^{-10}	77	31.7/8.48	4	1	14
16	1.55	6.04×10^{-4}	γ 3-Immunoglobulin	gi 1628395	99	2.70×10^{-5}	52	38.7/8.37	3	2	9
17	1.65	4.15×10^{-2}	Fibrinogen- γ	gi 223170	88	3.20×10^{-4}	NA	33.6/5.98	5	2	22
18	3.88	3.03×10^{-2}	Fibrinogen fragment D	gi 2781208	545	4.20×10^{-50}	165	38.1/5.84	22	3	62
19	4.59	3.97×10^{-2}	Macrophage capping protein	gi 21730367	136	3.30×10^{-9}	85	38.8/5.32	3	2	12
20	-2.08	3.38×10^{-2}	Ig γ 1	gi 226787	128	1.20×10^{-6}	47	25.6/7	4	1	21
21	-2.14	8.32×10^{-3}	Ig γ 1	gi 226787	148	1.20×10^{-8}	47	25.6/7	5	1	26
22	-1.74	2.69×10^{-2}	Ig γ 1	gi 226787	129	1.70×10^{-8}	48	25.6/7	4	1	21
23	-2.13	1.64×10^{-2}	Ig γ 1	gi 5031410	123	6.60×10^{-8}	59	25.4/6.95	3	0	15
24	-2.87	2.1×10^{-2}	Glyceraldehyde 3-phosphate dehydrogenase	gi 7669492	199	1.70×10^{-15}	98	36.2/8.57	5	0	22
25	-2.67	4.94×10^{-2}	Complement component C4A	gi 443671	430	1.30×10^{-38}	315	195.2/6.79	17	5	8
26	-1.91	3.62×10^{-2}	Phosphatidylinositol transfer protein	gi 5453908	129	1.70×10^{-8}	59	32/6.11	4	0	13
27	-1.58	3.71×10^{-2}	Thrombospondin	gi 553801	216	3.30×10^{-17}	138	42.2/6.6	5	0	14
28	19.01	8.49×10^{-3}	Fibrinogen α -chain	gi 76779196	279	1.70×10^{-23}	100	29.2/9.51	12	5	34
29	7.72	1.84×10^{-2}	Fibrinogen α -chain	gi 76779196	718	2.10×10^{-67}	399	29.2/9.51	21	6	58
30	1.78	1.22×10^{-2}	Peroxiredoxin 1	gi 55959888	134	3.10×10^{-7}	63	10.7/8.79	3	0	34
31	10.61	3.69×10^{-2}	Transgelin	gi 48255905	184	5.30×10^{-14}	74	22.7/8.87	6	2	34
32	-3.15	3.23×10^{-2}	Haptoglobin- α	gi 296653	237	2.60×10^{-19}	139	42.1/6.25	7	3	20

*Protein spot number according to Figure 1 and average volume ratio (luminal/abluminal) as quantified by DeCyder.

†Protein ID and accession number according to the National Center for Biotechnology Information database.

‡Mascot score, expected value, and ion score. NA indicates not applicable. Supplemental Figure I provides annotated MALDI-MS spectra. Ion score applies to peptide MS/MS fragmentation spectra.

§Number of peptides matching and not matching the protein sequence and protein sequence coverage.

nents), extracellular matrix remodeling (thrombospondin), hemostasis (eg, fibrinogen), or heme-related proteins (eg, hemopexin).

PRX-1 Is Mainly Localized and Released by the Luminal Part of AAA Thrombus

Among the differential proteins identified by 2D-DIGE and MS that were found to be altered in the 4 patients and not

previously related to AAA, we focused on PRX-1 because regulation of oxidative stress is of major importance in AAA pathophysiology. We validated the levels of PRX-1 in the conditioned media from the same samples used in the DIGE experiment (n=4) and from an additional group of human AAA samples (n=6). PRX-1 release was increased in the luminal layer of the ILT of AAA compared with the abluminal layer, as assessed by ELISA (32 ± 10 versus 10 ± 2 ng/mL,

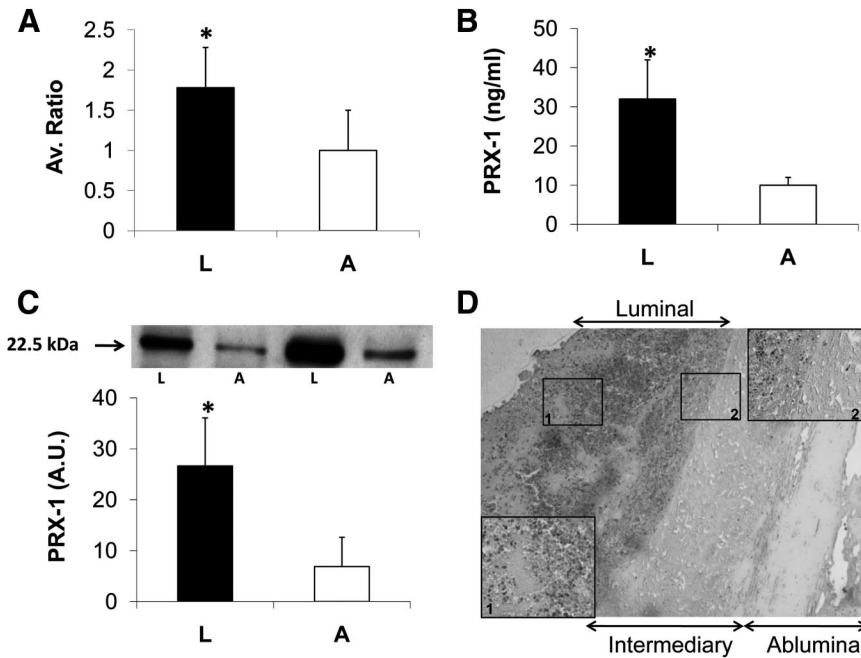


Figure 2. PRX-1 is expressed and released by the luminal part of AAA thrombus. A, Average spot volumes corresponding to luminal (L) ($n=4$) and abluminal (A) ($n=4$) samples are shown from the DeCyder software (*average ratio=1.78; $P<0.05$). B, Quantification of PRX-1 levels by ELISA measured in L and A layer supernatants ($n=10$, $*P<0.001$). C, Representative Western blot from L and A layer supernatants ($n=10$, $*P<0.001$). D, Immunodetection of PRX-1 in AAA thrombus (magnification, $\times 10$). Inset 1 (magnification, $\times 20$) shows erythrocyte accumulation with leukocyte (nucleated cells) in the luminal part of the thrombus. Inset 2 (magnification, $\times 20$) shows erythrocytes and polynuclear cells positive for PRX-1 at the interface between the luminal and intermediary layers of the thrombus. Positivity is shown in green and nuclei in red. A.U. indicates arbitrary units.

$P<0.05$) and Western blot (Figure 2). Immunodetection of PRX-1 in AAA thrombus (Figure 2D) showed a strong staining in the luminal layer mainly associated with RBCs and polymorphonuclear neutrophils.¹⁷ Both cellular and diffuse staining was observed, which suggests a potential release of PRX-1 in the extracellular compartment. On the contrary, the abluminal layer exhibited a faint staining, in accordance with results obtained by 2D electrophoresis, Western blot, and ELISA.

PRX-1 Is Induced and Released by Oxidative Stress in RBCs

Because the main function of PRX-1 is the inactivation of hydrogen peroxide (H_2O_2), we analyzed H_2O_2 levels in AAA thrombus-conditioned media. Interestingly, H_2O_2 levels were also increased in the luminal layer versus the abluminal layer (17.2 ± 5.4 versus 6.4 ± 1.4 $\mu\text{mol/L}$, $P<0.05$). In addition, in vitro experiments with isolated RBCs stimulated with H_2O_2 (and lipopolysaccharide) were performed. We have shown that H_2O_2 and lipopolysaccharide induced PRX-1 translocation to the membrane and final release to the conditioned medium (Figure 3). These results suggest that PRX-1 is released as a response to prooxidant molecules present in the thrombus.

Increased PRX-1 Levels in Serum of AAA Patients

This ex vivo approach offers the advantage of identifying proteins potentially released into the bloodstream that could serve as circulating biomarkers for AAA. We measured circulating PRX-1 levels in the serum of patients and controls by ELISA. Results showed that PRX-1 levels were significantly increased in patients with AAA relative to control subjects (14.9 ± 3.5 versus 8 ± 0.6 ng/mL, $P<0.001$, Figure 4A). Because AAA diameter is a surrogate marker of the growth rate, we studied the correlation between circulating PRX-1 levels and AAA diameter. Interestingly, a significant

positive correlation between PRX-1 levels and AAA diameter was found ($r=0.6$, $P<0.001$, Figure 4B).

Plasmin-antiplasmin (PAP) complexes and myeloperoxidase (MPO) levels are increased in patients with AAA.^{18,19} We confirmed these data in our patient population (data not shown). In addition, we have previously shown that both PAP and MPO levels are increased in the luminal part of the thrombus.^{18,19} Interestingly, PRX-1 positively correlated with

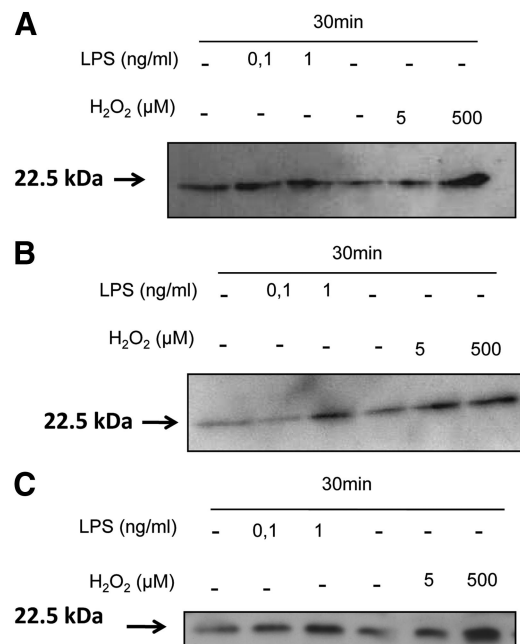


Figure 3. Western blot analysis of PRX-1 in RBCs. Representative Western blots were performed in RBCs isolated from healthy controls and stimulated with lipopolysaccharide (LPS) and H_2O_2 for 30 minutes at different concentrations. A, Total cell lysates. B, Supernatant obtained from cell culture. C, Membrane fractions.

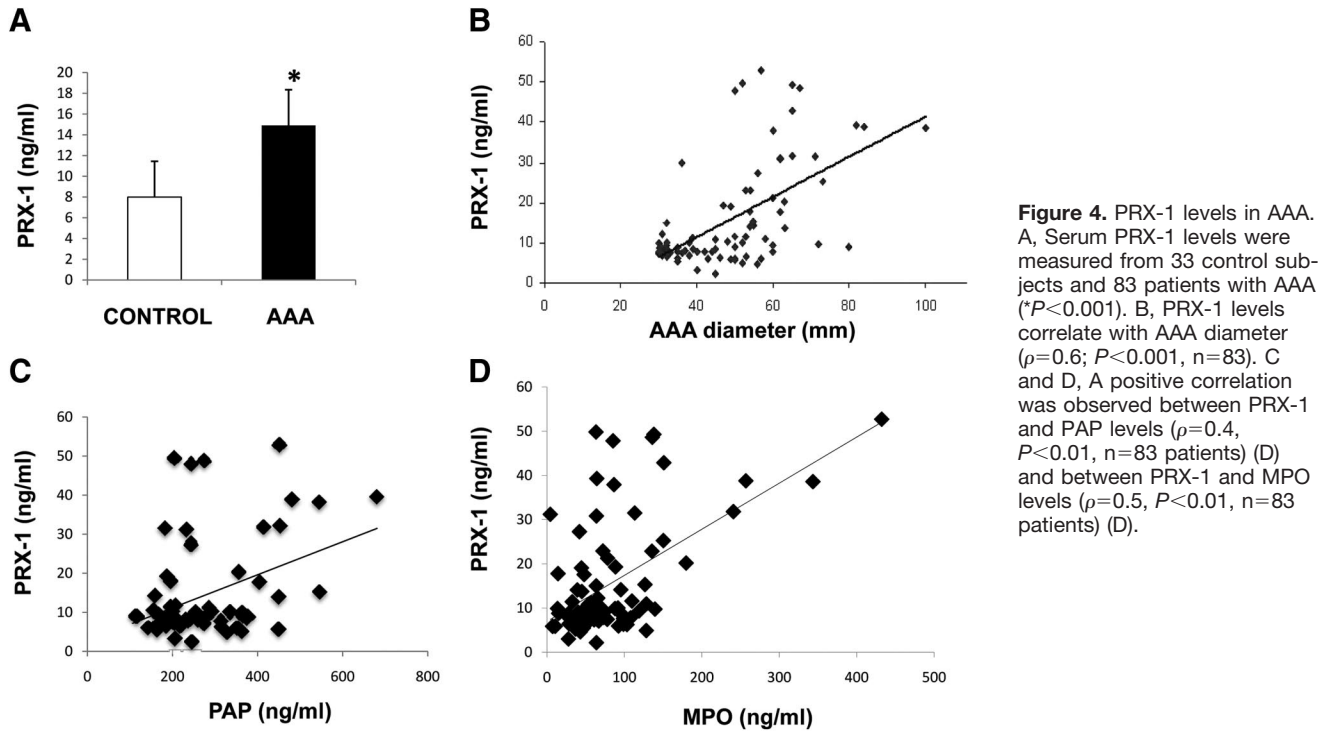


Figure 4. PRX-1 levels in AAA. A, Serum PRX-1 levels were measured from 33 control subjects and 83 patients with AAA (* $P<0.001$). B, PRX-1 levels correlate with AAA diameter ($\rho=0.6$; $P<0.001$, $n=83$). C and D, A positive correlation was observed between PRX-1 and PAP levels ($\rho=0.4$, $P<0.01$, $n=83$ patients) (D) and between PRX-1 and MPO levels ($\rho=0.5$, $P<0.01$, $n=83$ patients) (D).

both biomarkers of AAA in our patient population (PAP, $r=0.4$; MPO, $r=0.55$; $P<0.05$ for both; Figure 4C and 4D).

PRX-1 Circulating Levels Correlate With AAA Growth

To further extend the results obtained, we measured PRX-1 serum levels in a second cohort of 80 patients from the Viborg study, with a 5-year follow-up, allowing us to test the relevance of PRX-1 as a marker of AAA progression. Similar levels of circulating PRX-1 in small AAA patients (AAA size

3 to 5.5 cm) were obtained in both the Viborg and Spanish studies (Viborg= 10.4 ± 3.7 ng/mL versus small AAA Spanish= 8.3 ± 2.8 ng/mL). Interestingly, the correlation coefficient between PRX-1 levels and AAA growth rate was 0.3 ($P=0.01$, Figure 5A), persisting after adjustment for current smoking, body mass index, previous cardiovascular events, use of glucocorticoids, low-dose aspirin, β -blockers, angiotensin converting enzyme inhibitors, and ankle brachial blood pressure index (Supplemental Table III). ROC curve analyses showed that PRX-1 levels were equally valid predictors of

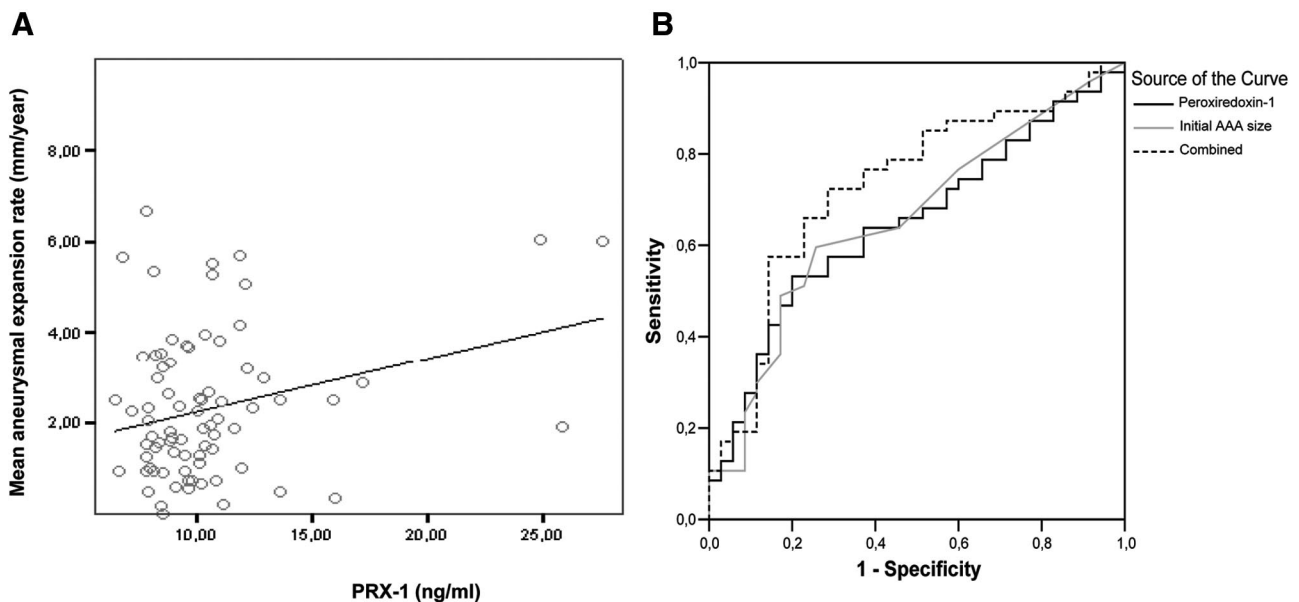


Figure 5. PRX-1 and AAA growth in the Viborg cohort. A, PRX-1 levels were measured from an additional group of serum samples (Viborg cohort). PRX-1 correlates with AAA growth ($\rho=0.3$; $P<0.05$, $n=80$). B, ROC curve for predicting AAA expansion concerning PRX-1 levels and AAA size.

annual expansion rate as initial AAA size exceeding the mean annual growth, with optimal cutoff points with a sensitivity and specificity of 63% and 64%, respectively, for both (Figure 5B). By combining these 2 predictors (PRX-1 and AAA-size) by linear regression to predict annual expansion rate, the optimal validity increased by almost 10% to 72% sensitivity and 71% specificity. Moreover, comparison of the C-statistics showed that the 3 ROC curves differed significantly (χ^2 test=9.11, $P=0.01$). Consequently, the combination of PRX-1 and size has significantly additive value in predicting growth compared with size alone.

Discussion

Vascular diseases are currently among the most common pathologies, and despite outstanding scientific advances in recent years, the number of effective therapies and useful biomarkers is still very limited, although there is a continuous increase in costs associated with these diseases. Although several potential biomarkers of AAA have been proposed,^{2,3} it is essential to discover new biomarkers for early disease detection and risk stratification, which could predict clinical outcome. Moreover, identification of novel biomarkers could help decipher the biological mechanisms leading to progressive dilatation and finally rupture to develop new therapeutic approaches. Our study was performed on the thrombus conditioned medium as a potential source of biomarkers of AAA by a gel-based proteomic approach using DIGE platform. Among the proteins identified by MS, both novel and previously known protein biomarkers for AAA have been unveiled that are associated with some major AAA pathological processes, such as thrombosis and oxidative stress.

The formation of a luminal thrombus may be considered a compensatory mechanism in response to flow perturbations associated with AAA dilatation. The mural thrombus is continuously self-forming at its luminal pole and subjected to proteolysis at the interface with the residual media. Previous studies have shown that thrombus formation and accumulation of leukocytes may have an impact on the structural integrity and stability of the vessel wall and thereby increase the risk of aneurysm rupture.²⁰ Proteins associated with thrombosis have been the biomarkers most commonly assessed in AAA. In this respect, levels of PAP complexes and D-dimers are elevated in AAA patients and correlate with disease progression.^{2,3} Similarly, elevated plasma fibrinogen concentrations predict a greater risk of thrombosis. Interestingly, fibrinogen levels are increased in AAA patients compared with controls, and positive correlations of AAA size, ILT, and fibrinogen concentration are observed.²¹ In the present work, among known biomarkers identified, we have shown that fibrinogen/fibrin fragments are abundantly released by the luminal part of the thrombus, in agreement with previous results of our group showing activation of the fibrinolytic system.¹⁹

Oxidative stress plays a key role in AAA pathophysiology.²² Although previous studies have mainly addressed the role of oxidative stress in AAA wall, the presence of a mural hemothrombus can also contribute to this process. The

trapping of RBCs within ILT may lead to hemolysis and subsequent release of the prooxidant hemoglobin that, when oxidized, transfers heme to endothelium and lipoproteins, thereby enhancing susceptibility to oxidant-mediated injury. Heme potentiates cell cytotoxicity mediated by leukocytes and other sources of reactive oxygen species.²³ Plasmatic hemopexin, haptoglobin, and albumin limit the hemoamplified oxidative damage to the vasculature. In the present work, a decrease in hemopexin and haptoglobin levels was observed in the luminal part of the thrombus, which could favor the prooxidant actions of hemoglobin.

However, oxidative stress is the result of imbalance between prooxidant and antioxidant molecules. In relation to antioxidant enzymes, superoxide dismutase, glutathione peroxidase, and thioredoxin expression levels are increased in AAA tissue.^{24,25} Thioredoxin, thioredoxin reductase, and NADPH together constitute a ubiquitous system that regulates cellular redox status. PRX-1 can interact and modulate NADPH activity by inactivating H_2O_2 .²⁶ In this respect, we observed that H_2O_2 levels were increased in the luminal layer versus the abluminal layer, similarly to the tendency observed for PRX-1. In addition, we have shown that H_2O_2 induced PRX-1 translocation to the membrane and final release to the conditioned medium in isolated RBCs. These results suggest that PRX-1 is released as a response to the prooxidant environment present in the ILT.

We hypothesized that the blood compartment could reflect what was observed in the arterial conditioned medium and thus that proteins potentially released into the circulation could serve as biomarkers for the pathology. We show here for the first time that PRX-1 levels are significantly increased in the serum of patients with AAA in relation to controls. Because AAA diameter is a surrogate marker of AAA growth rate and is the clinical parameter used in the management of AAA patients, we studied its potential correlation with PRX-1. We have observed that PRX-1 levels and AAA diameter show a significant positive correlation. Furthermore, a positive correlation among circulating PRX-1, PAP, and MPO levels has been shown, supporting the importance of ILT activities in AAA pathophysiology. Finally, PRX-1 also correlated with AAA growth in a second population with follow-up. Interestingly, by combining both AAA size with PRX-1 levels by linear regression to predict annual expansion rate above the interobserver variation of the measurements,¹⁶ the optimal validity increased almost by 10% up to 72% sensitivity and 71% specificity. The fact that PRX-1 correlates significantly with both AAA size and AAA growth rate in different populations (Spanish and Viborg studies), together with the observed comparable circulating levels in small AAA in the 2 populations, suggests the potential use of PRX-1 as a biomarker for AAA evolution.

On the other hand, despite the fact that PRX-1 levels could be increased in response to the oxidative stress present in AAA, the functional consequences of PRX-1 upregulation are not completely understood. In mammalian cells, high levels of peroxiredoxins are produced, which may account for 0.1% to 0.8% of soluble protein.²⁷ PRX-1

is overexpressed in response to oxidative stress and has recently been involved in other mechanisms, such as shear stress.²⁸ Several studies support an antioxidant and anti-apoptotic role for PRX-1 upregulation. PRX-1 was initially described mainly as an antioxidant protein because of its ability to inactivate H₂O₂, ONOO⁻, and other hydroperoxides. However, other cellular roles have been recently proposed for PRX-1, including the modulation of cytokine-induced H₂O₂ levels, which have been shown to mediate the signaling cascade that leads to cell proliferation, differentiation, and apoptosis,²⁹ and proinflammatory actions.³⁰ In this respect, PRX-1 has been shown to interact with other proteins and ligands, including hemo and macrophage migration inhibitor factor (MIF).^{27,31} MIF is upregulated in AAA³² and MIF serum levels have been correlated with annual AAA expansion rate and initial AAA size.³³ Interestingly, we have observed a significant strong positive correlation between PRX-1 and MIF in the Viborg sample cohort (not shown). MIF, by modulating the redox status of PRX-1, has been suggested to modulate signaling pathways and glucocorticoid sensitivity.³¹ Likewise, we have observed a positive correlation between PRX-1 levels and glucocorticoid treatment.

In the present study, several proteins associated with key mechanisms involved in AAA pathogenesis have been identified by a non-hypothesis-driven proteomic approach. Among them, increased PRX-1 levels have been observed both in conditioned media and in serum from AAA patients. PRX-1 serum levels are associated with AAA size and growth rate, suggesting its possible use as a biomarker for AAA evolution. Additional prospective studies are needed to confirm these results. These results support a main pathological role of oxidative stress in AAA,²² as well as the potential usefulness of therapies aimed at enhancing antioxidant pathways to prevent AAA progression.³⁴

Acknowledgments

We thank Dr Cesar Aparicio for the collection of human samples.

Sources of Funding

This work was supported by the Fighting aneurysmal disease project (FP-7, HEALTH F2-2008-200647 to J.B.M., J.S.L., J.E.), Ministerio de Ciencia e Innovacion (SAF2010/21852 to J.L.M-V., SAF2007/63648 to J.E.), Fundacion Ramon Areces (to L.M.B-C.), CAM (S2006/GEN-0247 to J.E.), Ministerio de Sanidad y Consumo, Instituto de Salud Carlos III, Redes RECAVA (RD06/0014/0035 to J.E.), PI10/00234 (to L.M.B-C.) and EUS2008-03565 (to J.E.) and Fundacion Pro CNIC (to J.A.L., E Calvo, E Camafeita).

Disclosures

None.

References

- Sakalihasan N, Limet R, Defawe OD. Abdominal aortic aneurysm. *Lancet*. 2005;365:1577–1589.
- Urbanavicius S, Urbanaviciene G, Honore B, Henneberg EW, Vorum H, Lindholt JS. Potential circulating biomarkers for abdominal aortic aneurysm expansion and rupture: a systematic review. *Eur J Vasc Endovasc Surg*. 2008;36:273–280.
- Golledge J, Tsao PS, Dalman RL, Norman PE. Circulating markers of abdominal aortic aneurysm presence and progression. *Circulation*. 2008;118:2382–2392.
- Blanco-Colio LM, López JA, Martínez-Pinna Albar R, Egido J, Martín-Ventura JL. Vascular proteomics, a translational approach: from traditional to novel proteomic techniques. *Expert Rev Proteomics*. 2009;6:461–464.
- Nordon I, Brar R, Hinchliffe R, Cockerill G, Loftus I, Thompson M. The role of proteomic research in vascular disease. *J Vasc Surg*. 2009;49:1602–16012.
- Blanco-Colio LM, Martín-Ventura JL, Muñoz-García B, Orbe J, Páramo JA, Michel JB, Ortiz A, Meilhac O, Egido J. Identification of soluble tumor necrosis factor-like weak inducer of apoptosis (sTWEAK) as a possible biomarker of subclinical atherosclerosis. *Arterioscler Thromb Vasc Biol*. 2007;27:916–922.
- Martin-Ventura JL, Duran MC, Blanco-Colio LM, Meilhac O, Leclercq A, Michel JB, Jensen ON, Hernandez-Merida S, Tuñón J, Vivanco F, Egido J. Identification by a differential proteomic approach of heat shock protein 27 as a potential marker of atherosclerosis. *Circulation*. 2004;110:2216–2219.
- Fontaine V, Touat Z, Mtairag el M, Vranckx R, Louedec L, Houard X, Andreassian B, Sebbag U, Palombi T, Jacob MP, Meilhac O, Michel JB. Role of leukocyte elastase in preventing cellular re-colonization of the mural thrombus. *Am J Pathol*. 2004;164:2077–2087.
- Caligiuri G, Rossignol P, Julia P, Groyer E, Mouradian D, Urbain D, Misra N, Ollivier V, Sapoval M, Boutouyrie P, Kaveri SV, Nicoletti A, Lafont A. Reduced immunoregulatory CD31+ T cells in patients with atherosclerotic abdominal aortic aneurysm. *Arterioscler Thromb Vasc Biol*. 2006;26:618–623.
- Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat Protoc*. 2006;1:2856–2860.
- Schuerenberg M, Luebbert C, Eickhoff H, Kalkum M, Lehrach H, Nordhoff E. Prestructured MALDI-MS sample supports. *Anal Chem*. 2000;72:3436–3442.
- Suckau D, Resemann A, Schuerenberg M, Hufnagel P, Franzen J, Holle A. A novel MALDI LIFT-TOF/TOF mass spectrometer for proteomics. *Anal Bioanal Chem*. 2003;376:952–965.
- Perkins DN, Pappin DJ, Creasy DM, Cottrell JS. Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis*. 1999;20:3551–3567.
- Vega de Ceniga M, Esteban M, Quintana JM, Barba A, Estallo L, de la Fuente N, Vivien B, Martin-Ventura JL. Search for serum biomarkers associated with abdominal aortic aneurysm growth: a pilot study. *Eur J Vasc Endovasc Surg*. 2009;37:297–299.
- Lindholt JS, Juul S, Fasting H, Henneberg EW. Screening for abdominal aortic aneurysms: single centre randomised controlled trial. *BMJ*. 2005;330:750.
- Lindholt JS, Vammen S, Juul S, Henneberg EW, Fasting H. The validity of ultrasonographic scanning as screening method for abdominal aortic aneurysm. *Eur J Vasc Endovasc Surg*. 1999;17:472–475.
- Neumann CA, Krause DS, Carman CV, Das S, Dubey DP, Abraham JL, Bronson RT, Fujiwara Y, Orkin SH, Van Etten RA. Essential role for the peroxiredoxin Prdx1 in erythrocyte antioxidant defence and tumour suppression. *Nature*. 2003;424:561–565.
- Houard X, Touat Z, Ollivier V, Louedec L, Philippe M, Sebbag U, Meilhac O, Rossignol P, Michel JB. Mediators of neutrophil recruitment in human abdominal aortic aneurysms. *Cardiovasc Res*. 2009;82:532–541.
- Houard X, Rouzet F, Touat Z, Philippe M, Dominguez M, Fontaine V, Sarda-Mantel L, Meulemans A, Le Guludec D, Meilhac O, Michel JB. Topology of the fibrinolytic system within the mural thrombus of human abdominal aortic aneurysms. *J Pathol*. 2007;212:20–28.
- Kazi M, Thyberg J, Religa P, Roy J, Eriksson P, Hedin U, Swedenborg J. Influence of intraluminal thrombus on structural and cellular composition of abdominal aortic aneurysm wall. *J Vasc Surg*. 2003;38:1283–1292.
- Al-Barjas HS, Ariens R, Grant P, Scott JA. Raised plasma fibrinogen concentration in patients with abdominal aortic aneurysm. *Angiology*. 2006;57:607–614.
- McCormick ML, Gavrilu D, Weintraub NL. Role of oxidative stress in the pathogenesis of abdominal aortic aneurysms. *Arterioscler Thromb Vasc Biol*. 2007;27:461–469.
- Balla J, Vercellotti GM, Jeney V, Yachie A, Varga Z, Jacob HS, Eaton JW, Balla G. Heme, heme oxygenase, and ferritin: how the vascular endothelium survives (and dies) in an iron-rich environment. *Antioxid Redox Signal*. 2007;9:2119–2137.

24. Dubick MA, Keen CL, DiSilvestro RA, Eskelson CD, Ireton J, Hunter GC. Antioxidant enzyme activity in human abdominal aortic aneurysmal and occlusive disease. *Proc Soc Exp Biol Med*. 1999;220:39–45.
25. Martinez-Pinna R, Lindholt JS, Blanco-Colio LM, Dejouvencel T, Madrigal-Matute J, Ramos-Mozo P, Vega de Ceniga M, Michel JB, Egido J, Meilhac O, Martin-Ventura JL. Increased levels of thioredoxin in patients with abdominal aortic aneurysms (AAA): a potential link of oxidative stress with AAA evolution. *Atherosclerosis*. 2010;212:333–338.
26. Leavey PJ, Gonzalez-Aller C, Thurman G, Kleinberg M, Rinckel L, Ambruso DW, Freeman S, Kuypers FA, Ambruso DR. A 29-kDa protein associated with p67phox expresses both peroxiredoxin and phospholipase A2 activity and enhances superoxide anion production by a cell-free system of NADPH oxidase activity. *J Biol Chem*. 2002;277:45181–45187.
27. Wood ZA, Schroder E, Harris JR, Poole LB. Structure, mechanism and regulation of peroxiredoxins. *Trends Biochem Sci*. 2003;28:32–40.
28. Mowbray AL, Kang DH, Rhee SG, Kang SW, Jo H. Laminar shear stress up-regulates peroxiredoxins (PRX) in endothelial cells: PRX 1 as a mechanosensitive antioxidant. *J Biol Chem*. 2008;83:1622–1627.
29. Wood ZA, Poole LB, Karplus PA. Peroxiredoxin evolution and the regulation of hydrogen peroxide signaling. *Science*. 2003;300:650–653.
30. Riddell JR, Wang XY, Minderman H, Gollnick SO. Peroxiredoxin 1 Stimulates Secretion of Proinflammatory Cytokines by Binding to TLR4. *J Immunol*. 2010;184:1022–1030.
31. Kudrin A, Ray D. Cunnin factor: macrophage migration inhibitory factor as a redox-regulated target. *Immunol Cell Biol*. 2008;86:232–238.
32. Verschuren L, Lindeman JH, van Bockel JH, Abdul-Hussien H, Kooistra T, Kleemann R. Up-regulation and coexpression of MIF and matrix metalloproteinases in human abdominal aortic aneurysms. *Antioxid Redox Signal*. 2005;7:1195–1202.
33. Pan JH, Lindholt JS, Sukhova GK, Baugh JA, Henneberg EW, Bucala R, Donnelly SC, Libby P, Metz C, Shi GP. Macrophage migration inhibitory factor is associated with aneurysmal expansion. *J Vasc Surg*. 2003;37:628–635.
34. Grigoryants V, Hannawa KK, Pearce CG, Sinha I, Roelofs KJ, Ailawadi G, Deatrick KB, Woodrum DT, Cho BS, Henke PK, Stanley JC, Eagleton MJ, Upchurch GR. Tamoxifen up-regulates catalase production, inhibits vessel wall neutrophil infiltration, and attenuates development of experimental abdominal aortic aneurysms. *J Vasc Surg*. 2005;41:108–114.

Arteriosclerosis, Thrombosis, and Vascular Biology



JOURNAL OF THE AMERICAN HEART ASSOCIATION

Identification of Peroxiredoxin-1 as a Novel Biomarker of Abdominal Aortic Aneurysm

Roxana Martinez-Pinna, Priscila Ramos-Mozo, Julio Madrigal-Matute, Luis M. Blanco-Colio, Juan A. Lopez, Enrique Calvo, Emilio Camafeita, Jes S. Lindholt, Olivier Meilhac, Sandrine Delbosc, Jean-Baptiste Michel, Melina Vega de Ceniga, Jesus Egido and Jose L. Martin-Ventura

Arterioscler Thromb Vasc Biol. 2011;31:935-943; originally published online January 27, 2011;
doi: 10.1161/ATVBAHA.110.214429

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272
Greenville Avenue, Dallas, TX 75231

Copyright © 2011 American Heart Association, Inc. All rights reserved.

Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the
World Wide Web at:

<http://atvb.ahajournals.org/content/31/4/935>

Data Supplement (unedited) at:

<http://atvb.ahajournals.org/content/suppl/2011/01/27/ATVBAHA.110.214429.DC1.html>

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Arteriosclerosis, Thrombosis, and Vascular Biology* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the [Permissions and Rights Question and Answer](#) document.

Reprints: Information about reprints can be found online at:
<http://www.lww.com/reprints>

Subscriptions: Information about subscribing to *Arteriosclerosis, Thrombosis, and Vascular Biology* is online at:
<http://atvb.ahajournals.org/subscriptions/>

SUPPLEMENTAL MATERIAL

SUPPLEMENTARY METHODS

In-gel trypsin digestion

Protein spots from silver-stained gels were visually matched against DIGE images, manually excised from gels, and transferred to pierced V-bottom 96-well polypropylene microplates (Bruker Daltonik, Bremen, Germany) loaded with ultrapure water. Samples were digested automatically using a Proteineer DP protein digestion station (Bruker Daltonik) according to the protocol of Schevchenko et al. (1) with minor variations: gel plugs were submitted to reduction with 10 mmol/l DTT (GE Healthcare, Uppsala, Sweden) in 50 mmol/l ammonium bicarbonate (99.5% purity; Sigma Chemical, St. Louis, MO, USA) and alkylation with 55 mmol/l iodoacetamide (Sigma Chemical) in 50 mmol/l ammonium bicarbonate. Gel pieces were then rinsed with 50 mmol/l ammonium bicarbonate and acetonitrile (gradient grade; Merck, Darmstadt, Germany) and dried under a nitrogen stream. Modified porcine trypsin (sequencing grade; Promega, Madison, WI, USA) at a final concentration of 7.5 ng/ μ l in 50 mmol/l ammonium bicarbonate was added to the dry gel pieces, and digestion proceeded at 37°C for 10 h. The resulting digestion solutions were transferred by centrifugation to V-bottom 96-well polypropylene microplates (Greiner Bio-One, Frickenhausen, Germany), vacuum-dried and kept at 4°C for later MS analysis.

MALDI Mass spectrometry

Dried samples were redissolved in 0.2 g/l α -cyano-4-hydroxycinnamic acid (Bruker Daltonik) in 50% aqueous acetonitrile and 0.2% trifluoroacetic acid (99.5% purity; Sigma Chemical). This solution was deposited onto a 600 μ m AnchorChip prestructured MALDI probe (Bruker Daltonik) (2) and allowed to dry at room temperature. Samples were

automatically analyzed in an Ultraflex MALDI-TOF/TOF mass spectrometer (Bruker Daltonik) (3) with an automated analysis loop controlled by the flexControl 2.2 software (Bruker Daltonik). In a first step, MALDI-MS spectra were acquired by averaging 400 individual spectra in the positive ion reflector mode at 50 Hz laser frequency in a mass range from 800 to 3500 Da. Internal calibration of MALDI-MS mass spectra was performed using two trypsin autolysis ions with $m/z = 842.510$ and $m/z = 2211.105$. In a second step, precursor ions showing in the MALDI-MS mass spectrum were subject to fragment ion analysis in the tandem (MS/MS) mode to average 1200 spectra. For MALDI-MS/MS, calibrations were performed with fragment ion spectra obtained for the proton adducts of a peptide mixture covering the 800-3200 m/z region. Automated analysis of mass data was performed using the flexAnalysis 2.2 software (Bruker Daltonik). No smoothing or any further spectral processing was applied. MALDI-MS and MS/MS spectra were manually inspected in detail and reacquired, recalibrated and/or relabeled using the aforementioned programs and homemade software when necessary.

MALDI-MS Database searching

MALDI-MS and MS/MS data were combined through the BioTools 3.0 program (Bruker Daltonik) to search a nonredundant protein database (NCBI nr 20091015, $\sim 10^7$ entries, National Center for Biotechnology Information, Bethesda US), using the Mascot software v2.2 (Matrix Science, London, UK; <http://www.matrixscience.com>) (4). Other relevant search parameters were set as follows: enzyme, trypsin; fixed modifications, carbamidomethyl (C); allow up to 1 missed cleavage; peptide tolerance ± 20 ppm; MS/MS tolerance ± 0.5 Da. Protein scores greater than 82 were considered significant ($p < 0.05$).

References:

1. Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat Protoc.* 2006; 1: 2856-2860.
2. Schuerenberg M, Luebbert C, Eickhoff H, Kalkum M, Lehrach H, Nordhoff E. Prestructured MALDI-MS sample supports. *Anal Chem.* 2000; 72: 3436-3442.
3. Suckau D, Resemann A, Schuerenberg M, Hufnagel P, Franzen J, Holle A. A novel MALDI LIFT-TOF/TOF mass spectrometer for proteomics. *Anal Bioanal Chem* 2003; 376: 952-965
4. Perkins DN, Pappin DJ, Creasy DM; Cottrell JS. Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis.* 1999; 20: 3551-3567.

TABLE I (online). *Characteristics of the patients included in the Spanish Cohort Study*

	N	%
Previous hypertension	50	55
Previous dyslipemic	56	65
Previous diabetes	11	14
Previous cardiovascular diseases	32	36
Past/present smokers	41	46
Use of aspirin	33	37
Use of statin	50	55
Use of Ace inhibitors	37	42

TABLE II (online). *Characteristics and events of the included men from The Viborg Cohort Study*

	N	%	
Previous hypertension*	10	12.8	
Previous cerebral ischaemic attack*	4	5.1	
Previous angina pectoris*	12	15.4	
Previous acute myocardial infaction*	20	25.6	
Previous lower limb ischaemia*	5	6.4	
Current smokers	45	57.7	
Use of low dose aspirin	37	47.4	
Use of statin	0	0.0	
Use of Betablockers	15	19.2	
Use of Ace inhibitors	10	12.8	
Planned repair due to progression	16	20.5	
Dead	37	47.4	
	N	Mean	SD
Age at inclusion	78	68.1	2.9
Systolic brachial blood pressure (mmHg)	78	157.8	21.6
Diastolic brachial blood pressure (mmHg)	78	92.6	13.2
Ankle brachial blood pressure index	78	0.99	0.24
Body Mass Index (kg/m ²)	78	27.0	3.9
Initial AAA-size (mm)	78	33.4	4.0
Annual aneurismal growth rate (mm/year)	78	2.22	1.56

* Identified by national-wide registry of hospital-diagnoses at discharge from hospital

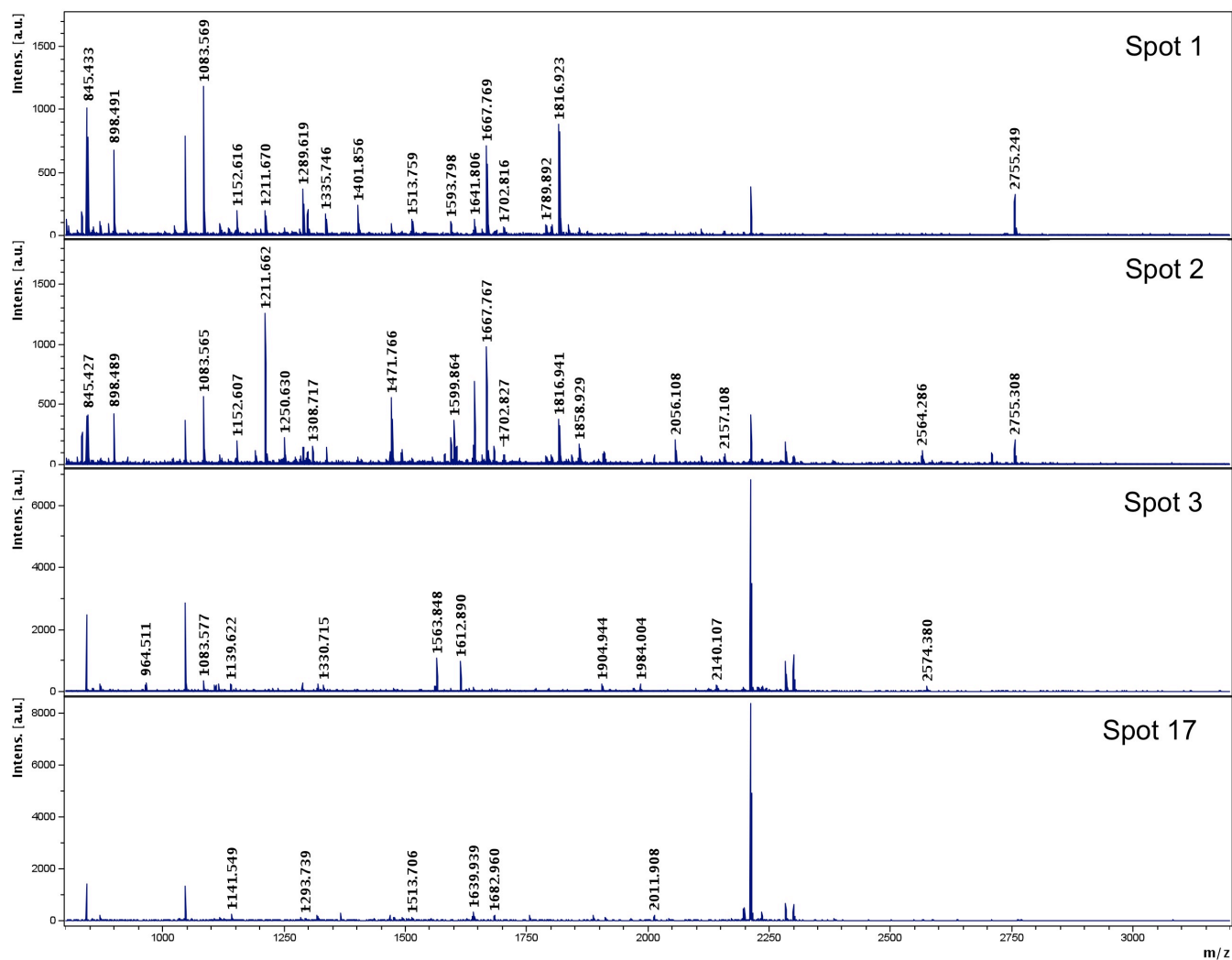
Table III (online). *Multivariate analysis performed on Viborg cohort study*

Model	Coefficients(<i>a</i>)			t	Sig.
	Unstandardized Coefficients		Standardized Coefficients		
	B	Std. Error	Beta		
(Constant)	-4,997	2,096		-2,384	0,02
Peroxiredoxin-1	0,106	0,048	0,251	2,21	0,031
Initial AAA-size	0,142	0,045	0,354	3,147	0,002
Current smoking	0,936	0,378	0,287	2,48	0,016
Use of glucocorticoids	-0,344	0,58	-0,069	-0,593	0,555
Body mass index	0,001	0,047	0,001	0,013	0,99
Coexisting cardiovascular disease	0,348	0,409	0,107	0,85	0,398
Ankle brachial bloodpressure index	0,783	0,748	0,117	1,047	0,299
Use of β -blocker	-0,167	0,516	-0,041	-0,323	0,747
Use of ACE-inhibitor	0,468	0,56	0,098	0,836	0,406
Use of low-dose aspirin	-0,048	0,374	-0,015	-0,128	0,899

a Dependent Variable: Mean annual aneurismal expansion rate (mm/year)

SUPPLEMENTARY FIGURE.

Supplementary Figure I. MALDI-MS spectra corresponding to proteins identified without MS² data.



Supplementary Figure 1. MALDI-MS spectra corresponding to proteins identified without MS² data.